PERSPECTIVE

Polymerase Chain Reaction

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Advances in nucleic acid technology during the past five years have yielded practical probe-based assays for diagnosing infectious and genetic diseases. Although the assays are sensitive, cells, tissues, or body fluids often must be cultured to increase the cell or organism number to readily detectable levels, yet culture is not always easy or successful.

A novel technique, the polymerase chain reaction (PCR), was recently developed for in vitro amplification of the DNA or RNA of an organism or gene defect, and culture may not be required. The PCR takes advantage of an enzyme that uses a defined segment in a strand of DNA as a template for assembling a complementary strand.

The principle of the PCR is simple, requiring a three-step cycling process: (*1*) denaturation of double-stranded DNA, (*2*) annealing of primers, and (*3*) primer extension. If an RNA sequence is to be amplified, a DNA copy of it (cDNA) must be synthesized by using reverse transcriptase before the PCR is begun. A cycle typically takes ~3–5 min and is repeated 20–40 times [1]. The PCR reaction vessel contains a mixture of buffers, nucleotides, primers, enzyme, and nucleic acid from the specimen of interest.

Denaturation separates the complementary strands of DNA held together in the duplex by hydrogen bonds. Although there are several physical and chemical means of dissociating the duplex, heating it to 95–100 C is simple and efficient.

In the annealing process, primers are attached to the dissociated DNA strands. A primer is a single-stranded sequence of nucleotides known as an oligonucleotide. Each primer is complementary to one of the original DNA strands, to either the left (5') or right (3') side of the sequence of interest. The primers are present in such vast molar excess that they are more likely to anneal to the dissociated strands than the strands are to reanneal to each other.

Once annealing has occurred, enzyme catalyzes the synthesis of new strands of DNA. The enzyme is a DNA polymerase that adds nucleotides complementary to those in the unpaired DNA strand onto the annealed primer. The number of DNA strands doubles upon completion of each cycle. After 30 cycles, a single copy of DNA can be increased up to 1 000 000 copies. In a sense, then, the replication of a discrete strand of DNA is being manipulated in a tube under controlled conditions.

In early PCR experiments, the enzyme used was the Klenow fragment of *Escherichia coli* DNA polymerase I, but this heat-labile enzyme had to be added in each subsequent cycle because the enzyme was inactivated during each denaturation step. The Klenow fragment now has been replaced by the thermostable DNA polymerase of *Thermus aquaticus* (Taq), a development that has permitted automation of the procedure because all reaction components can be combined at the beginning. In addition, use of Taq polymerase has improved the specificity, yield, sensitivity, and length of target DNA that can be amplified [2].

Amplified sequences of target DNA can be detected by a variety of methods. If enough amplified DNA is present, it can be visualized after gel electrophoresis and ethidium bromide staining; however, this method cannot provide definitive identification of the product. This is particularly true if the target sequence initially is present in extremely small quantities, a situation that may lead to amplification of some nonspecific sequences and yield too little specific DNA to be visualized. Therefore, final detection assays usually rely on hybridization of a portion of the amplified DNA product to specific synthetic DNA probes representing a portion of the amplified sequences.

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Oligomer restriction (OR) is a common detection method. It has been applied in our laboratory and others to detect amplified DNA of the human immunodeficiency virus (HIV) [3–6] and human T cell lymphotropic virus type I (HTLV-I) [7–9].

Amplified DNA is hybridized in solution to a synthetic DNA probe that is labeled at its 5' end with a radioactive phosphate group [10]. The probe also possesses a restriction enzyme recognition site known to be present in the amplified DNA. Hybridization of the single-stranded probe to the amplified DNA restores the recognition site to a double-stranded form. Subsequent digestion with the restriction enzyme releases the radiolabeled fragment of the DNA probe. This fragment has a known length and is easily detected by polyacrylamide gel electrophoresis and autoradiography. One limitation of OR is that mutation of the target sequence (sequence diversity) could lead to loss of the recognition site. Abbott et al. [7] in this issue of the *Journal* recommend the use of slot blotting and hybridization to avoid the problems associated with sequence diversity.

The PCR has rapidly become a valuable technique for molecular biologists. Marx [11] recently has reviewed a number of applications of the PCR in a variety of settings. Wooly mammoth mitochondrial DNA and DNA from the brain of an Egyptian mummy have been amplified and sequenced [11]. Mitochondrial DNA from single hairs has been detected by Higuchi et al. [12], a finding making PCR a useful tool in forensic medicine. Phenylketonuria [13] and α-1-antitrypsin deficiency [14] have been added to the list of genetic diseases amenable to study by the PCR. Miyano et al. [15] have used the PCR to screen for a point mutation not associated with a restriction fragment length polymorphism in the insulin gene. Crescenzi et al. [16] have detected one lymphoma cell in 10^5 normal cells, an observation revealing the subclinical presence of leukemic cells in patients thought to be in complete remission and in bone marrow evaluated for autologous transplantation.

Furthermore, the PCR technique shows tremendous promise in the study of viral diseases, particularly those for which serology or culture are difficult and where early detection of infection may have tremendous value in public health. Shibata et al. [17] have used the PCR to detect human papilloma virus (HPV) in paraffin-embedded tissue. In addition, the PCR has proved to be extremely useful for detecting HIV and HTLV. Amplified DNA of HIV type 1 (HIV-1) and HTLV-I have been detected directly in peripheral blood mononuclear cells of seropositive persons [3, 8, 18, 19].

Although licensed tests are available to detect antibody to HIV-1, none are available for detecting antibodies to HIV type 2 (HIV-2; endemic in West Africa) or HTLV-I or HTLV type II. In addition, there are no licensed assays for detecting HIV or HTLV antigens, and culture is notoriously difficult and labor-intensive. The sensitivity of culture for HIV-1 varies among laboratories, although Jackson et al. [20] recently reported recovery of the virus from 99% of infected individuals in a single cohort of 140 homosexual men and two women seen in their AIDS Clinical Trial Unit in Minnesota.

A rapid and sensitive assay for HIV or HTLV would provide a better understanding of their pathogenesis. For example, what is the meaning of an indeterminate western blot? Why do some individuals at high risk for HIV infection remain seronegative and asymptomatic? Does active replication or transcriptional dormancy correlate with the antibody or antigen picture? Is drug therapy (e.g., zidovudine [formerly known as AZT]) effective in inhibiting viral replication and transcription in vivo, and does it reduce the virus burden in an infected person? These questions and others reflect major areas of research related to HIV being pursued by using the PCR.

Diagnosing HIV infection in infants born to seropositive mothers is difficult because maternal antibody can persist for up to 15 mo. The PCR has been used to detect HIV DNA in some of these infants [21, 22]. Further study may provide useful information for identifying those infants with a poor prognosis or those eligible for antiviral therapy.

HIV DNA has been detected by the PCR at least six months before western blot–confirmed seroconversion [23]. Because RNA sequences can be detected in cells of HIV-infected persons by amplifying cDNA copies of reverse-transcribed cellular RNA, it is possible to differentiate latent HIV infection (proviral DNA) from active viral transcription (RNA) [4, 24, 25]. Furthermore, detecting HIV RNA and DNA in the cells and body fluids of individuals before and after seroconversion may help to explain latency and define the relation between cell-associated and cell-free virus [5].

There have been occasional reports of positive antibody tests reverting to negative. Farzadegan et al.

[26] have used the PCR to demonstrate the presence of HIV-1 DNA in four men who had seroreverted out of 1000 asymptomatic homosexual men tested. Despite repeated attempts, the investigators did not succeed in culturing virus from cells of these men. The significance of these results remains to be determined.

Rayfield et al. [27], as reported in this issue of the *Journal*, recently have used the PCR to differentiate between HIV-1 and HIV-2. In addition, they demonstrated concurrent infection with HIV-1 and HIV-2 in an individual. The PCR was also used to confirm the first case of HIV-2 infection in a West African living in the United States [28].

Kwok et al. [29] in this issue of the *Journal* report the direct cloning and sequencing of amplified HTLV-I DNA, and the same approach has been applied to HIV RNA in our laboratory [4]. This represents a considerable reduction in the time otherwise required to obtain the same information by conventional techniques. In addition, it is a major improvement in the determination of viral sequences. Because direct amplification, cloning, and sequencing does not require cultivation of viruses in vitro, artificial selection of strains able to grow in these conditions is eliminated. As a result, sequence data obtained from this approach will more accurately reflect the viral sequences actually present in patients.

Because of the exquisite sensitivity of the PCR, it seems to be ideally suited to monitoring the effect of antiviral therapy [4, 25]. Also, by amplifying a dilution series of known positive DNA simultaneously, it is possible to quantify the virus burden by determining the number of proviral copies per given number of cells [4, 7, 19].

The research summarized here merely suggests the power of the PCR. Its power is, however, its own weakness. Even the smallest amount of contaminating DNA can be amplified, resulting in misleading or ambiguous results. To minimize the chance of contamination, investigators should reserve separate rooms for reagent preparation, the PCR, and analysis of product. Reagents should be dispensed into single-use aliquots. Pipetters with disposable plungers and extra-long pipette tips should be routinely used.

Another important consideration is the use of controls and what those controls should be. Ideally, their matrix should be identical to that of the experimental system, and they should be included in every run. Positive and negative controls must be clearly distinguishable; their failure invalidates the experiment and should warn the investigator of a problem somewhere in the system.

Sensitive and specific nonradiometric detection methods for PCR products are being developed. The PCR amplifies target sequences not the detection system. Boosting the number of target sequences improves the performance of less-sensitive nonradiometric probes and may yield practical screening methods for early viral infection.

The PCR is a powerful and practical research tool. The heretofore unknown etiologies of many diseases are being clarified by the PCR. The technique may enable the identification of previously unknown viruses related to those already known. If the procedure can be further simplified and sensitive nonradiometric detection systems can be developed, the PCR will assume a prominent place in the clinical laboratory.

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